Immunohistochemical Localization of Glutamate Decarboxylase in Rat Cerebellum

(Purkinje cells/ γ -aminobutyric acid/synapse)

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ABSTRACT Glutamate decarboxylase (L-glutamate 1-carboxylase; EC 4.1.1.15), the enzyme in brain that forms γ -aminobutyric acid, was made visible on sections of rat cerebellum by use of rabbit antiserum to purified mouse-brain glutamate decarboxylase. Cerebellar sections obtained from rats that were perfused with 4% paraformaldehyde were treated with antiserum against the enzyme or with serum from unimmunized rabbits, washed, and then incubated with peroxidase-labeled goat antibody against rabbit immunoglobulin. The glutamate decarboxylase was made visible on sections by means of the product formed by the action of peroxidase on 3,3'-diaminobenzidine and H_2O_2 . A weak and diffuse reaction was observed in Purkinje cell bodies, suggesting the occurrence of the enzyme within these cells. In addition, an intense, punctate deposition of reaction product was located around the Purkinje cells and around the neurons of the deep cerebellar nuclei, suggesting the impingement of many nerve terminals containing the enzyme upon these neuronal surfaces. No specific reaction product was observed in sections treated with serum from unimmunized rabbits. The distribution of glutamate decarboxylase observed in our preparations is consistent with a large body of indirect biochemical, physiological, and morphological data dealing with the synaptic role of γ -aminobutyric acid neurons in the cerebellum.

The first report of the presence of γ -aminobutyric acid (GABA) in uniquely large concentrations in vertebrate central nervous system was made in 1950 (1). As a result of much work in several laboratories it now appears probable that GABA is an inhibitory transmitter at the crustacean neuromuscular junction, and it seems possible that it is a major inhibitory transmitter in the vertebrate central nervous system. Work with subcellular fractionation procedures, using electron microscopic monitoring, has shown that L-glutamate decarboxylase (L-glutamate 1-carboxylase; EC 4.1.1.15; GAD), the enzyme that catalyzes the α -decarboxylation of L-glutamate to form GABA and CO_2 , is particularly concentrated in presynaptic nerve-ending fractions (see refs. 2 and 3 for suitable references). One of our major goals has been to localize the formation and action of GABA at precise cellular sites in the vertebrate nervous system. Toward this end, mouse-brain GAD has now been purified to homogeneity (4), and antisera

to the enzyme which crossreact with GAD from several species (5) have been produced in rabbits. In the present communication we will describe the immunohistochemical localization of GAD in rat cerebellum using antibody against the purified mouse-brain enzyme.

MATERIALS AND METHODS

Rabbit Antiserum. Antiserum to GAD purified from mouse brain (anti-GAD serum) was produced in rabbits by biweekly injections of 150 μ g of GAD in complete Freund's adjuvant into the infrascapular muscles. The antiserum in this experiment was from a rabbit that had received 600 μ g of GAD over an 8-week period. This antiserum reacted only with GAD in the crude enzyme preparation from brain, and some of its properties have been reported previously (5).

Peroxidase-Labeled Antibody to Rabbit Immunoglobulin G (IgG). Peroxidase-labeled antibody against rabbit IgG from goat was prepared according to the procedure of Nakane *et al.* (6). Goat IgG (110 mg) against rabbit IgG (Miles Lab.) and 110 mg of horseradish peroxidase (Sigma, Type VI) were dissolved in 4 ml of 1 M carbonate buffer (pH 10.0), and 0.5 ml of 0.5% *p,p'*-difluoro-*m-m'*-dinitrophenyl sulfone (Sigma Chemical Co.) in acetone was added. The mixture was stirred gently for 8 hr at 4° and was then dialyzed against phosphate-buffered saline (PBS). The protein was precipitated by addition of an equal volume of a saturated ammonium sulfate solution. The precipitate was dissolved in a small volume of PBS and was reprecipitated by addition of an equal volume of a saturated ammonium sulfate solution. This procedure was performed four more times, after which the resulting solution was fractionated on a Bio-Gel P-300 column, eluting with PBS. Conjugates that were both immunologically and enzymatically active were eluted in fractions subsequent to enzymatically active but immunologically inactive ones.

Absorption by Acetone Powder. In order to reduce nonspecific reaction involving staining of antigen in tissue section, each 0.5-ml portion of antiserum from immunized rabbits or serum from unimmunized rabbits was absorbed with 100 mg of PBS-washed mouse-liver powder (acetone-dried, Miles Lab.) at room temperature for 30 min. Peroxidase-labeled conjugates were absorbed with 100 mg of PBS-washed mouse-brain powder (acetone-dried, Miles Lab.) at room temperature for 30 min.

Abbreviations: GAD, glutamate decarboxylase; GABA, γ -aminobutyric acid; PBS, phosphate-buffered saline, pH 7.2, containing 75 mM NaCl and phosphate; IgG, immunoglobulin G.

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Preparation of Brain Sections. Adult Sprague-Dawley rats were anesthetized by intraperitoneal injection of 35% chloral hydrate (1 ml/kg of body weight) and then perfused through the heart for 15 min with a fixative composed of 4% paraformaldehyde in 0.12 M Millonig's phosphate buffer, pH 7.2 (7). After the brain was fixed *in situ* for 1 hr at room temperature, the cerebellum was removed, sectioned sagittally into two pieces, and placed overnight at 4° in the same fixative as that used for the perfusion. The fixed cerebellum was mounted in 5% agar and cut into 75- μ m parasagittal sections with a Sorvall TC-2 tissue sectioner. The sections were collected in cold 0.12 M Millonig's buffer.

Staining Procedures. Unmounted sections (approximately 0.25 cm²) were incubated for 30 min at room temperature with

0.3 ml of absorbed anti-GAD serum or serum from unimmunized rabbits, both of which were diluted to $\frac{1}{8}$ the original concentration with PBS containing IgG from unimmunized goats to give a final concentration of 1 mg/ml. The sections were washed with PBS for 3 hr at room temperature, and then were incubated with peroxidase-labeled antibody against rabbit IgG prepared in goat (1.2 mg/ml) at room temperature for 30 min. After they were washed with PBS for 3 hr. at room temperature, the sections were incubated with 3,3'-diaminobenzidine·4HCl (30 mg/50 ml of PBS, Sigma Chemical Co.) and H₂O₂ (0.006%) at room temperature for 10 min, and washed with PBS overnight at 4°. The sections then were osmicated with 1% OsO₄, dehydrated, and mounted. On focusing the microscope through the thickness of sections

FIG. 1. (A) Control section of a cerebellar folium and nucleus interpositus. Molecular layer (m), Purkinje layer (p), granular layer (g), nucleus interpositus (N.I.). No specific reaction product is observed in this section. (B) Experimental section of a cerebellar folium and nucleus interpositus. Purkinje cells (long arrows) show a heavy reaction. The molecular layer (m) shows a lighter, more diffuse reaction and the granular layer (g) (short arrows) has a less-heavy punctate reaction product distributed throughout. The nucleus interpositus (N.I.) shows dense reaction product throughout its volume.

treated with immune serum, the impression was gained that the reaction product appeared only about one-third of the way into the sections from either side.

RESULTS

Even in low-magnification light micrographs, striking differences were observed between control cerebellar sections (Fig. 1*A*) and experimental sections treated with antibody against mouse-brain GAD prepared in rabbit (Fig. 1*B*). For example, a specific, highly localized staining reaction was observed to be associated with the Purkinje cells and in the granule cell layer of sections treated with anti-GAD serum, while a more

diffuse stain appeared in the molecular layer of such preparations. In addition, the deep cerebellar nucleus contained within our sections, the nucleus interpositus, stained very heavily and was bordered by unstained white matter. No specific localization of reaction product was observed when serum from unimmunized rabbits was used.

Dense staining in a punctate distribution around neuronal somata and their proximal dendrites was found in the nucleus interpositus upon examination at higher magnification of the sections treated with anti-GAD serum (Fig. 2*B*), but not in the controls (Fig. 2*A*). The bouton-like nature of these punctate, positive sites was enhanced by use of Nomarski optics (Fig.

FIG. 2. (A) Control section of nucleus interpositus. Neuronal soma (s). (B) Neuropil of nucleus interpositus from an experimental preparation. Soma of neuron (s), dendrite (d), reaction product (long arrows), grazed neuron soma (encircled by short arrows) with bouton-like reaction product on cell surface (b). (C) Neuron shown in Fig. 2*B*, photographed with Nomarski optics. Soma (s), dendrite (d), bouton-like deposits of reaction product (b).

FIG. 3. (A) Photomicrograph of a cerebellar folium from an experimental preparation. Granule cell layer (G), Purkinje cell body (P), molecular layer (M), Purkinje cell dendrite (d), reaction product corresponding to expected distribution of basket-cell axonal endings (long black and white arrows); reaction product within the granule-cell layer which may be marking Golgi type-II axonal endings upon granule-cell dendrites (short black arrows); reaction product along dendrite of Purkinje cell (long white arrows); reaction product not associated with obvious structures (short white arrows). (B) Nomarski optic photomicrograph of a cerebellar folium from a control preparation. Purkinje cell body (P), granule cell layer (G). (C) Nomarski optic photomicrograph of experimental folium. Purkinje cell body (P), granule cell layer (G), reaction product resembling basket endings on Purkinje cell soma (arrows).

2C). When neuronal somata were grazed by the blade of the tissue sectioner, a dense packing of bouton-like structures was clearly visible over the somal surface (Fig. 2B). The bouton-like structures have diameters (about 1–2 μm), which are consistent with the size of Purkinje-cell axon terminals in synaptic contact with deep cerebellar neurons (8). In addition, the pattern of reaction product deposition seen in Figs. 2B and 2C is very similar to the distribution of degenerated Purkinje cell terminals in the cat nucleus interpositus after cerebellar cortical lesions (see Fig. 1 in ref. 8). High-magnification light-microscopic examination revealed a diffuse staining of the cytoplasm of Purkinje cells (Fig. 3A and 3C) that was either absent or much less intense in the Purkinje-cell cytoplasm of control preparations (Fig. 3B). Moreover, there were numerous punctate deposits of reaction product around the somata of the Purkinje cells in sections treated with anti-GAD serum, reaction sites which correspond to the expected distribution of basket-cell endings. Generally smaller and lighter punctate deposits were seen throughout the molecular layer, and some of these deposits were clearly distributed along Purkinje-

cell dendrites (Figs. 3A and 3C). Such deposits of reaction product may correspond to the endings of stellate cells on Purkinje-cell dendrites or, perhaps, to the endings of the Purkinje-cell recurrent collaterals forming the supraganglionic plexus (9). The deposition of reaction product within the granular layer (Fig. 3A) might be marking the axonal endings of Golgi type-II neurons upon the dendrites of granule cells.

DISCUSSION

Biochemical analytical data have shown the presence of GAD and GABA in many regions of the vertebrate central nervous system. However, in no instance have the functional relationships been worked out to the same extent as they have been in the cerebellum (2, 3). GABA probably mediates the inhibitory actions of stellate and basket cells upon the Purkinje cells and of the Golgi type-II cells upon granule-cell dendrites. It also probably transmits the inhibitory messages that go from the Purkinje cells to each other and possibly to other cerebellar cortical cells, as well as to the cells in the deep cerebellar nuclei. The present communication contains the

first report of an immunohistochemical method for the visualization of GAD at the cellular level. The data obtained to date are in keeping with the above concepts of the role of GABA in cerebellar function, but they are not proof. The techniques must be extended to the visualization of the punctate deposits of reaction product at the electron microscopic level in order to determine whether or not such a deposition occurs specifically within the boutons of likely GABA neurons. Furthermore, it will be necessary to study the consequences of a variety of experimental manipulations before the role of GABA as a transmitter at specific cellular sites can be stated with certainty.

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